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Review

Targeting the hypoxic response in bone tissue engineering: A balance between supply and consumption to improve bone regeneration

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ABSTRACT

Bone tissue engineering is a promising therapeutic alternative for bone grafting of large skeletal defects. It generally comprises an *ex vivo* engineered combination of a carrier structure, stem/progenitor cells and growth factors. However, the success of these regenerative implants largely depends on how well implanted cells will adapt to the hostile and hypoxic host environment they encounter after implantation. In this review, we will discuss how hypoxia signalling may be used to improve bone regeneration in a tissue-engineered construct. First, hypoxia signalling induces angiogenesis which increases the survival of the implanted cells as well as stimulates bone formation. Second, hypoxia signalling has also angiogenesis-independent effects on mesenchymal cells *in vitro*, offering exciting new possibilities to improve tissue-engineered bone regeneration *in vivo*. In addition, studies in other fields have shown that benefits of modulating hypoxia signalling include enhanced cell survival, proliferation and differentiation, culminating in a more potent regenerative implant. Finally, the stimulation of endochondral bone formation as a physiological pathway to circumvent the harmful effects of hypoxia will be briefly touched upon. Thus, angiogenic dependent and independent processes may counteract the deleterious hypoxic effects and we will discuss several therapeutic strategies that may be combined to withstand the hypoxia upon implantation and improve bone regeneration.

1. Introduction

Tissue engineering aims to offer large-scale replacement of damaged organs using implants generated from a combination of cells, growth factors and an appropriate scaffold. The engineering of bone tissue is a promising therapeutic strategy for non-healing large bone defects, as autologous bone transplants, the current gold standard, are limited in quantity and often associated with donor-site morbidity. A key challenge in the clinical application of cell-based tissue-engineered bone implants is the poor diffusion of oxygen into avascular tissue, which is limited to 200 μm at most, necessitating a functional blood vessel network to bring oxygen into larger constructs (Ma et al., 2014b). However, the blood vessels at the implant site are in most cases disrupted by the trauma or surgery that caused the bone defect, leading to hypoxia or even anoxia within the implant. This impaired oxygenation and nutrient supply may lead to low cell survival and failure of the implant. Indeed, studies in cardiac tissues have shown that tissue-engineered constructs of clinically relevant size are devoid of oxygen in the centre, causing massive loss of cell viability and regenerative capacity (Radisic et al., 2006). Analogous herewith, necrotic cells are observed in the centre of implanted large bone constructs (Scheufler et al., 2008). On the other hand, bone cells have the capacity to withstand the temporary hypoxia that occurs during certain stages of bone development or uncomplicated fracture repair (Drager et al., 2015) by activating the hypoxia signalling pathway. Indeed, a recent study has shown that in bone, physiological oxygen concentrations can be as low as 1.3% or 10 mmHg (Spencer et al., 2014), which are much lower than the ambient oxygen levels that we breathe (21% or 160 mmHg). Moreover, activation of the hypoxia signalling pathway is known to increase exponentially as oxygen concentrations fall below 8% or 60 mmHg (Ehrismann et al., 2007). A better understanding of the processes activated by the hypoxia signalling pathway in skeletal cells may therefore provide novel options to improve bone tissue engineering strategies.

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In this review, we will discuss the current knowledge on hypoxia signalling in bone regeneration and we will provide an overview of the different strategies that are being explored in bone tissue engineering to overcome cell death after implantation due to the long-lasting and extensive hypoxia, including pro-angiogenic therapies, prevascularization strategies and cellular preconditioning.

2. Bone tissue engineering

While bone has a tremendous regeneration potential and is one of the few tissues that can heal completely without scar formation, 10% of fractures results in a non-union (Tytherleigh-Strong et al., 1997). In case of congenital defects, like neurofibromatosis 1, or surgical resection, this regenerative potential may become even more challenged and often leads to inadequate healing, which causes an enormous health care burden. The current gold standard treatment consists of bone auto- or allografts, but several problems restrict their usefulness including the limited availability at the harvest site, incomplete integration into the defect and risk of disease transfer (Delloye et al., 2007). A promising therapeutic alternative for autografts in large bone defects is bone tissue engineering, which is a branch of regenerative medicine that aims to create new bone tissue from osteogenic stem or progenitor cells seeded on an osteoconductive carrier or scaffold, often in combination with osteoinductive growth factors.

The bone-forming cells frequently used for tissue engineering are the mesenchymal stromal cells (MSC, also known as mesenchymal stem cells). Bone marrow-derived MSC are used most often, although it are the periosteum-derived cells that primarily contribute to fracture healing and are therefore increasingly being studied (Colnot, 2009; Gomez-Barrena et al., 2011). Indeed, human and mouse periosteum-derived cells were considered much more difficult to obtain than bone marrow-derived cells, but protocols for their isolation have been developed and can now be routinely used (De Bari et al., 2006; van Gastel et al., 2012). These cells highly express classical MSC markers, show trilineage potential *in vitro* and contribute extensively to bone regeneration *in vivo*, making them exceptionally suited for bone tissue engineering strategies (Roberts et al., 2015). Adipose-derived MSC and embryonic or induced pluripotent stem cells are also being actively explored as cell source because of their greater availability. However, their *in vivo* bone-forming potential has not been as rigorously studied as for the bone-derived MSC (reviewed in (Robey, 2011)).

Numerous materials are used as carriers for these cells: they range from biological materials designed to closely mimic the composition of bone (i.e. calcium phosphate and collagen) over synthetic polymers (e.g. polylactic acid, polycaprolactone) to bioactive glass and metals, and many types of composite designs. Most scaffold materials can be chemically treated or biologically functionalized with peptides to enhance their osteoconductivity, degradability and angiogenic response. As the focus of this review is on the hypoxic response as a potential therapeutic target, we refer the reader for more information on biomaterial characteristics and their functionalization to some recent excellent reviews (Bose et al., 2012; Wu et al., 2014).

Finally, certain growth factors that have been described to be crucial for fracture healing are also being used for bone tissue engineering (reviewed in (Gothard et al., 2014)). Perhaps the most important examples are the bone morphogenic proteins (BMPs), notably BMP-2. They are currently approved for spinal fusion and open tibia fractures, although application of high-dosed BMP alone has proven not to be as safe as initially believed (Shields et al., 2006). Angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or placental growth factor, are also crucial for fracture repair and can be used to induce angiogenesis in bone tissue-engineered constructs. Several other factors, like fibroblast growth factors or transforming growth factors, can act on both osteo- and angiogenesis (reviewed in (Stegen et al., 2015)). However, when these factors are used as monotherapy for bone repair, they often have to be applied in high doses and frequently result in adverse side effects such as soft tissue inflammation and swelling, haematoma formation, heterotopic ossification and induction of osteolysis. These complications seriously limit their clinical applicability (Epstein, 2011; Shields et al., 2006). Nevertheless, these growth factors may potentially be used at much lower doses when they are combined with cell-based constructs. It is hypothesized that this reduced dose may be sufficient to stimulate primarily the bone forming potential of the implanted cells, although supporting clinical evidence is still lacking. Alternatively, the use of growth factors can be restricted to the pre-implantation cell expansion phase in order to enhance the regenerative potency of the implanted MSC and thereby prevent possible side effects caused by *in vivo* application of growth factors (van Gastel et al., 2014).

To summarize, bone tissue engineering is an expansive field with new combination approaches being developed continuously. However, the therapeutic success of clinically relevant regenerative implants will largely depend on how the implanted cells are adapted to react to the hostile and hypoxic host environment into which they are placed.

3. The hypoxia signalling pathway

The crucial mediators of hypoxia signalling are Hypoxia Inducible Factor 1 (HIF-1) and HIF-2, heterodimeric transcription factors consisting of an α and β subunit. Both subunits are constitutively expressed in most tissues and cells, but the HIF-α subunit is turned over very rapidly at the protein level under normoxic conditions (Fig. 1). When sufficient oxygen is available, HIF-1α is hydroxylated by proline hydroxylase domain (PHD)-containing enzymes (PHD1, PHD2, PHD3), of which PHD2 appears to be the main effector for HIF-1 activity (Appelhoff et al., 2004; Berra et al., 2003). These enzymes use oxygen and α-ketoglutarate as substrates and ferrous iron and ascorbate as cofactors. Upon hydroxylation of HIF-1α, the Von Hippel-Lindau protein (VHL) ubiquitinates the N- and C-terminal oxygen dependent degradation domains of HIF-1α, causing HIF-1α to be targeted for proteasomal degradation (Maxwell et al., 1999). As a result, HIF-1α is a highly unstable protein in aerobic conditions. When oxygen tension decreases, the PHDs will become less active, resulting in stabilization of HIF-1α which can then bind to HIF-β and exert its transcriptional activity in the nucleus (Huang et al., 1998). Reduced activity of PHDs through lowered oxygen concentration is however not the only mechanism that can induce HIF stabilization. Certain hormones and growth factors, such as angiotensin II, epidermal growth factor and insulin-like growth factor can induce HIF-mediated signalling in normoxic conditions via activation of protein kinase C and phosphatidylinositol-3 kinase (Fukuda et al., 2002; Richard et al., 2000; Zhong et al., 2000). Interestingly, the osteochondrogenic Runt-related transcription factor 2 (Runx2) also directly increases HIF-1α activity in MSC and hypertrophic chondrocytes by competing with VHL (Kwon et al., 2011; Lee et al., 2012). Hypoxia signalling can also be chemically induced by using small molecules that block PHD activity; most of them interfere with cofactor or substrate availability (e.g. iron chelators, α-ketoglutarate analogues) and therefore not only target PHDs, but specific PHD inhibitors are actively being developed.

The HIF complex transactivates genes containing one or more hypoxia response elements (5′-[A/G]CGTG-3′), of which more than 100 have been identified to date. These genes are mainly involved in the regulation of energy metabolism, angiogenic response,

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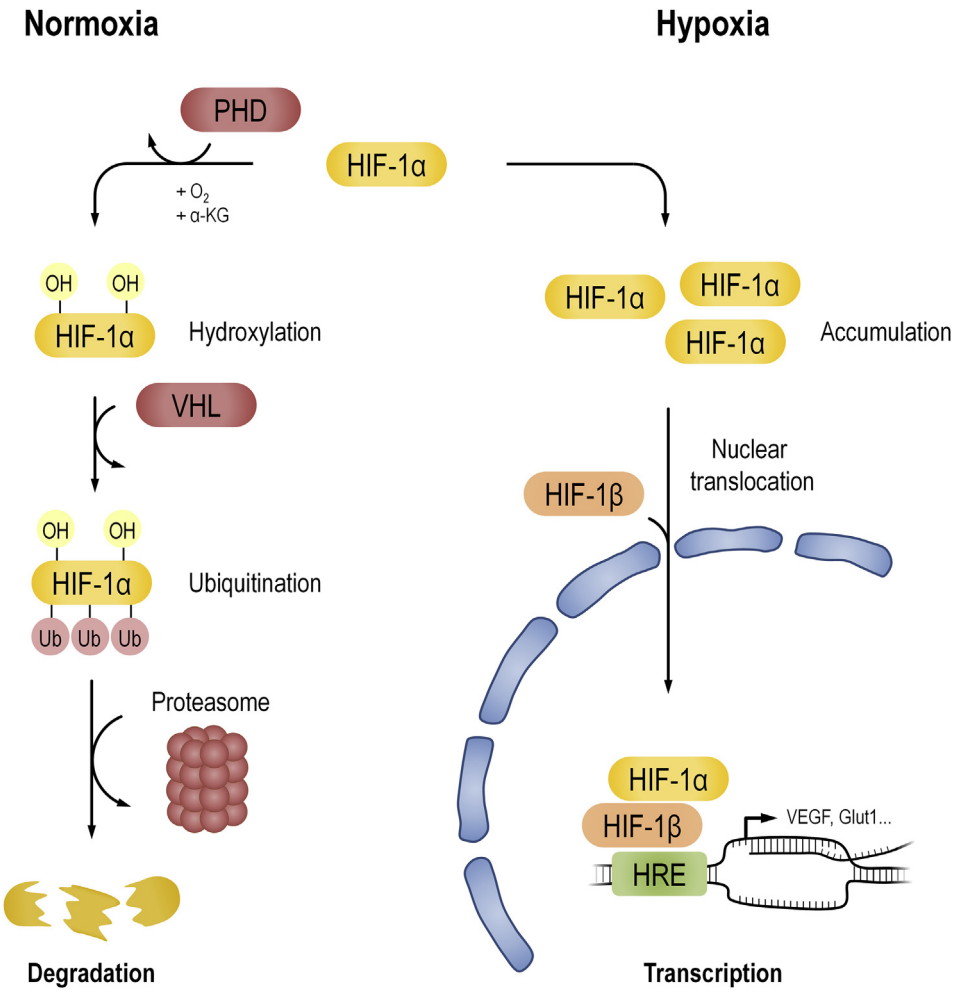

Fig. 1. Overview of the hypoxia signalling cascade mediated by Hypoxia Inducible Factor 1α (HIF-1α). Under normoxia, HIF-1α is rapidly hydroxylated by proline hydroxylase domain (PHD)-containing enzymes which use oxygen (O_2) and α-ketoglutarate (α-KG) as substrates. HIF-1α is then ubiquitinated (Ub) by interaction with the Von Hippel-Lindau protein (VHL), and is targeted for proteosomal degradation. When oxygen tension decreases, PHDs become inactive and HIF-1α will accumulate and translocate to the nucleus, where it can exert its transcriptional activity together with HIF-1β by binding to hypoxia response elements (HRE) in genes like glucose transporter 1 (Glut1) or vascular endothelial growth factor (VEGF).

erythropoiesis and cell survival; examples include glucose transporter 1, VEGF and erythropoietin. There are three known HIF-α subunits, of which HIF-1α is the best studied. HIF-2α undergoes the same proteolytic degradation and appears to regulate a set of genes overlapping with HIF-1α (Flamme et al., 1997), but it also controls specific target genes and differs from HIF-1α in its spatial expression pattern, indicating that HIF-2α may act on different processes (Ema et al., 1997; Hu et al., 2003; Wiesener et al., 2003). With respect to skeletal development and homeostasis, both factors have been shown to regulate these processes differentially (Saito et al., 2010; Shomento et al., 2010). HIF-3α, on the other hand, is not closely related to the others and functions mainly as a negative regulator of HIF-mediated gene expression as it lacks transcriptional activator domains (Makino et al., 2001).

4. Hypoxia in bone repair

Bone repair recapitulates many of the molecular pathways regulating bone development and these processes are also of interest to successfully engineer bone (Gerstenfeld et al., 2003). In all these instances, bone is formed either directly by committed osteoblast progenitors through intramembranous ossification, or via endochondral bone formation where a cartilage template is first formed. The chondrocytes in this template will produce a cartilaginous matrix and then hypertrophy and undergo apoptosis, which coincides with mineralization of the matrix and vascular invasion. Several genetic mouse studies have underscored the importance of HIF signalling in skeletal development and regeneration, both through its regulation of tissue vascularization, which we will discuss first, as well as through cell-autonomous effects discussed in a subsequent section.

During bone growth, conditional deletion of VHL in osteoblasts resulted in stabilization of HIF, enhanced VEGF expression, increased angiogenesis and a higher bone mass, whereas mice lacking HIF-1α in osteoblasts had reduced vasculature and bone volume (Wang et al., 2007). Upon fracture or trauma the blood supply to the injured bone is disrupted greatly, resulting in hypoxia in the surrounding cells and haematoma formation. These events, together with the elicited inflammatory reaction lead to a strong angiogenic response in the damaged tissue, which will ultimately secure the supply of oxygen, nutrients and growth factors (reviewed in (Stegen et al., 2015)). The importance of enhanced angiogenesis for bone repair has been evidently shown by different animal models and clinical observations (Brinker and Bailey, 1997;

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Fang et al., 2005; Glowacki, 1998; Hausman et al., 2001; Maes et al., 2006). We and others have shown that periosteum-derived cells are likely contributing to this reaction, as they are able to mount a strong pro-angiogenic and osteogenic response following exposure to hypoxia *in vitro* (Ichijima et al., 2012; van Gastel et al., 2012). In addition, activation of the HIF pathway also contributes to improved bone regeneration, as shown in a model of distraction osteogenesis, where enhanced hypoxia signalling in osteoblasts leads to better vascularization and bone regeneration in a VEGF-dependent manner (Wan et al., 2008). Moreover, compounds that induce hypoxia signalling are reported to promote fracture healing in rodents (Shen et al., 2009; Wan et al., 2008).

However, the effects of hypoxia signalling in bone development are likely not exclusively mediated via increasing angiogenesis, as has been shown in several developmental and *in vitro* studies. For instance, the growth plate is an avascular tissue and upon size expansion, the centrally localized chondrocytes need HIF signalling to adapt to and survive in the developing low-oxygen environment (Provot et al., 2007; Schipani et al., 2001). However, the HIF null mouse phenotype is only partially rescued by VEGF overexpression and the associated increase in angiogenesis, suggesting that other unidentified mechanisms are involved (Maes et al., 2012; Schipani et al., 2001). Consistent herewith, deletion of the soluble isoforms of VEGF, which are directly regulated by HIF, negatively affects chondrocyte survival in the hypoxic centre, not only by delaying vascular invasion, but also in a cell-autonomous manner (Maes et al., 2004). A possible mechanism is that HIF signalling in chondrocytes regulates the expression of collagen processing enzymes, a pathway that may contribute to preservation of extracellular matrix production and indirectly to chondrocyte survival (Aro et al., 2012).

Numerous *in vitro* studies have reported on the direct, angiogenesis-independent, role of hypoxia signalling for the differentiation of MSC to osteoblasts and for the function of mature osteoblasts, but data are inconsistent (an overview of recent literature is provided in Table 1). The differences in oxygen level, duration and timing of hypoxia during cell culture, cell source and cell parameters studied may explain the variability in cellular responses observed between the different studies. It is therefore difficult to draw a conclusion from the plethora of data reported, but in general, *in vitro* models of moderate-low oxygen tension (3–5% oxygen) appear to correlate with positive effects on osteoblast proliferation and differentiation, whereas more severe hypoxia negatively regulates these osteoblast properties. Besides these *in vitro* data suggesting a cell-autonomous effect of hypoxia signalling in osteogenic cells, recent *in vivo* observations also point to direct changes in osteoblasts. When HIF signalling was induced in osteochondral progenitor cells or mature osteoblasts *in vivo*, the increase in bone mass was associated with an increase in the proliferation and number of osteoblasts, although these findings were not demonstrated to be angiogenesis-independent (Wang et al., 2007; Weng et al., 2014). Recent evidence highlights that metabolic adaptations in osteoblasts, resulting in activation of glycolysis, are essential for the increased bone formation when HIF-1α is stabilized in osteoprogenitors postnatally (Regan et al., 2014). Furthermore, HIF activation by deleting PHD2 and PHD3 in osteoprogenitors enhanced the expression of osteoprotegerin, an osteoclast-inhibiting factor, and the associated decrease in osteoclast formation resulted in increased accumulation of bone mass (Wu et al., 2015). However, one has to be cautious to extrapolate these findings to bone tissue engineering, as these studies were performed by activating HIF signalling in cells experiencing a well-vascularized environment. It remains therefore to be shown whether the HIF pathway will be sufficient for implanted cells to adapt to a microenvironment devoid of oxygen and/or nutrients, which they encounter after implantation.

Taken together, hypoxia signalling plays an important role in the formation, maintenance and regeneration of bone by angiogenesis-dependent and -independent mechanisms.

5. Hypoxia in bone tissue engineering

A timely and sufficiently strong hypoxia response is thus needed during bone repair to stimulate angiogenesis and bone formation. However, the process of angiogenesis is an inherently slow process (estimates range from 100 μm to 1 mm per day (Mikos et al., 1993; Orr et al., 2003)), and the timely generation of a stable vascular network has long been identified as a fundamental challenge for tissue engineering (Koike et al., 2004). Because of the inherent constraints of vascular ingrowth, constructs of clinically relevant size may progressively become devoid of oxygen and nutrients in their centre upon implantation, which will be detrimental for cellular function and survival (Radisic et al., 2006; Scheufler et al., 2008). For this reason, a major research focus lies on either increasing the availability of oxygen or reducing its need in large tissue engineering constructs.

6. Reducing hypoxia through extrinsic angiogenesis

As the lack of blood vessel perfusion is a main contributor to hypoxia in tissue-engineered constructs, speeding up the process of (re)vascularization is an attractive strategy to remedy this problem (Fig. 2A). Extrinsic or host-driven vascularization can be stimulated through local administration of VEGF (Takeshita et al., 1994). As it is the main angiogenic growth factor in fracture healing, VEGF has been shown to enhance bone regeneration (Street et al., 2002; Tarkka et al., 2003). However, safety concerns exist over the use of high-dosed VEGF (Lee et al., 2000; Maes et al., 2010; Vajanto et al., 2002), which may lead to fibrosis and abnormal blood vessel formation and function. Nevertheless, by optimizing this approach, VEGF can be used to improve angiogenesis in a bone tissue-engineered construct when applied in a more sustained, low-dose regimen. This strategy can be achieved either through functionalization of the scaffold with VEGF (Detsch et al., 2014; Jabbarzadeh et al., 2012) or through its genetically induced expression by the implanted cells (Feng et al., 2013; Helmrich et al., 2013). Besides VEGF, other factors like thrombin and pleiothropin appear to have beneficial effects. Indeed, data from *in vitro* experiments with thrombin and *in vivo* experiments with pleiothropin respectively indicate that these factors enhance the angiogenic and osteogenic response of mesenchymal cell lines and human bone-marrow derived MSC (Bluteau et al., 2006; Yang et al., 2003). Furthermore, adaptations to scaffold material, design and porosity can be used to stimulate blood vessel ingrowth. For instance, the pore size of the scaffold is an important property that influences the *in vivo* osteogenic response, where small pores (<100 μm) will result in a hypoxic environment that favours cartilage formation, whereas larger pores (>300 μm) are more readily vascularized and promote direct bone formation (Karageorgiou and Kaplan, 2005). Thus, adaptations in timing and dosing of administered angiogenic growth factors as well as optimization in scaffold properties may improve adequate and timely blood vessel ingrowth into the scaffold.

7. The potential of intrinsic angiogenesis to decrease hypoxia

Establishing a vascular network from within the construct, or intrinsic vascularization, is an alternative approach that may help overcome detrimental hypoxia in a tissue-engineered construct. Instead of relying on growth factors to attract blood vessels from

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Table 1
Overview of the effect of hypoxia on proliferation and differentiation of osteoblasts and osteoblast progenitors.

Reference	Cell type	Hypoxia model	Timing	Proliferation	ALP	Runx2	Col1	OCN	Mineralization
Primary osteoblasts (*in vitro*)									
Warren et al., 2001	rat calvaria OB	5% O_2	Expansion (48 h)				↑		
Utting et al., 2006	rat calvaria OB	2% O_2	Expansion (6-18 d)	↓					
		2% O_2	Differentiation (18 d)		↓				↓
Salim et al., 2004	mouse calvaria OB	2% O_2	Preconditioning (24 h)			=	=	=	=
		<0.1% O_2	Preconditioning (24 h)			↓	↓	↓	↓
Wang et al., 2007	mouse calvaria OB	VHL KO	Both (14 d)	=	=	=		=	=
Primary MSC (bone marrow-derived, *in vitro*)									
Boyette et al., 2014	human BMSC	5% O_2	Expansion (4 d)	↑					
		5% O_2	Differentiation (21 d)			↑	↑	↑	↑
Zhou et al., 2013	rat BMSC	5% O_2	Expansion (14 d)	↑	↑			↑	
Berniakovich and Giorgio, 2013	mouse BMSC	3% O_2	Both (21 d)	↑					↑
Fehrer et al., 2007	human BMSC	3% O_2	Both (21 d)	↑					↓
Wagegg et al., 2012	human BMSC	2% O_2, DFO, DMOG	Differentiation (28 d)			↑			↑
Tsai et al., 2011	human BMSC	1% O_2	Both (21 d)	↑					↑
Park et al., 2013	human BMSC	1% O_2	Both (28 d)	↑				↓	↓
Yang et al., 2011	human BMSC	1% O_2	Both (21 d)			↓			↓
Hsu et al., 2013	human BMSC	1% O_2	Differentiation (21 d)		↓	↓		↓	
Cicione et al., 2013	human BMSC	1% O_2	Differentiation (21 d)		↓				↓
Lee et al., 2015	human vertebral BMSC	1% O_2	Both (21 d)	↓	↓	↓			↑
Li et al., 2015	mouse BMSC	1% O_2	Expansion (24 h)	↑					
Xu et al., 2013	rat BMSC	1% O_2	differentiation (21 d)		↓	↓		↓	↓
Primary MSC (other sources, *in vitro*)									
Ichijima et al., 2012	rat calvaria PDC	5% O_2	Both (72 h)	=	↑	↑		↑	
Fotia et al., 2015	human adipose-derived MSC	3% O_2	Both (14 d)	↑	↑				↑
Zhang et al., 2014	human periodontal cells	2% O_2	Both (21 d)	↑					
Xu et al., 2014	rabbit adipose-derived MSC	1% O_2	Both (28 d)	↑	↑			↑	↑
Ding et al., 2014	rat adipose-derived MSC	DMOG	Both (21 d)	↓	↑	↑		↑	↑
Mesenchymal cell lines (*in vitro*)									
Chen et al., 2012	MC3T3	1% O_2	Expansion (72 h)	↓		Wnt signalling activity ↓			
Chen et al., 2013	MC3T3	1% O_2	Expansion (48 h)			SOST expression ↑			
Zhou et al., 2015	C3H10T1/2	HIF-1α overexpression	Differentiation (9 d)					↓	↓
Peng et al., 2014	C3H10T1/2	DMOG	Differentiation (21 d)		↑	↑			↑
Sugrue et al., 2014	commercial cell lines	5% O_2	Expansion (7 d)	↑					
Pattappa et al., 2013	commercial cell lines	5% O_2	Both (21 d)	↓	↓				↓
Volkmer et al., 2010	commercial cell lines	2% O_2	Both (21 d)	↑	↓				↓
***In vivo* genetic mouse models**									
Regan et al., 2014	OSX − HIF1-PPN	HIF-1α overexpression in osteoprogenitors	5w old	=	More osteoblasts				
Weng et al., 2014	Col2-cre VHL^{flox}	VHL KO in osteochondral progenitors	16w old	↑	More osteoblasts				
Wang et al., 2007	OCN-cre VHL^{flox}	VHL KO in mature OB	3w old		More osteoblasts				
Zuo et al., 2015	OCN-cre VHL^{flox}	VHL KO in mature OB	6w old	↑	β-catenin and Osterix expression ↑				

Abbreviations used: alkaline phosphatase (ALP), bone marrow-derived mesenchymal stromal cell (BMSC), type I collagen (Col1), type II collagen (Col2), deferoxamine (DFO), dimethyloxallyl glycine (DMOG), hypoxia inducible factor 1α (HIF-1α), knockout (KO), mesenchymal stromal cell (MSC), osteoblast (OB), osteocalcin (OCN), osterix (OSX), periosteum-derived cell (PDC), runt-related transcription factor 2 (Runx2), sclerostin (SOST), Von Hippel-Lindau protein (VHL). Timing reflects when and for how long in hours or days hypoxia was applied before analysis: short-term hypoxia (expansion), hypoxia only during osteogenic differentiation conditions but not during expansion (differentiation), hypoxia both during expansion and differentiation (both), and hypoxia during expansion but not subsequent differentiation (preconditioning). Effects are shown as increased (↑), decreased (↓) or unchanged (=) compared to controls.

the host into the implant, scaffolds can be microfabricated to contain small hollow fibres or capillary structures that mimic the morphology of blood vessels and may serve as a template for vascularization (Fidkowski et al., 2005; Moroni et al., 2006). Furthermore, instead of using only osteoblast progenitors, the additional incorporation of endothelial (progenitor) cells into tissue-engineered constructs is a possible approach (Fig. 2B). The idea behind this strategy is that when these two cell populations are seeded on a scaffold, the endothelial compartment will form a vascular network through so-called *in vitro* prevascularization. Upon implantation, this network then only has to anastomose to the host vasculature to establish perfusion. Indeed, many studies have shown that implanted endothelial cells can contribute to the blood vessels that are formed inside tissue-engineered constructs (Koike et al., 2004; Ma et al., 2014a; van Gastel et al., 2012). While promising, most approaches are still in an experimental stage with varying degrees of success, even in small defects (Kim and von Recum, 2008). An important consideration for the engineering of blood vessel networks is that they will face regression without adequate stabilization by mural cells (Gerhardt and Betsholtz,

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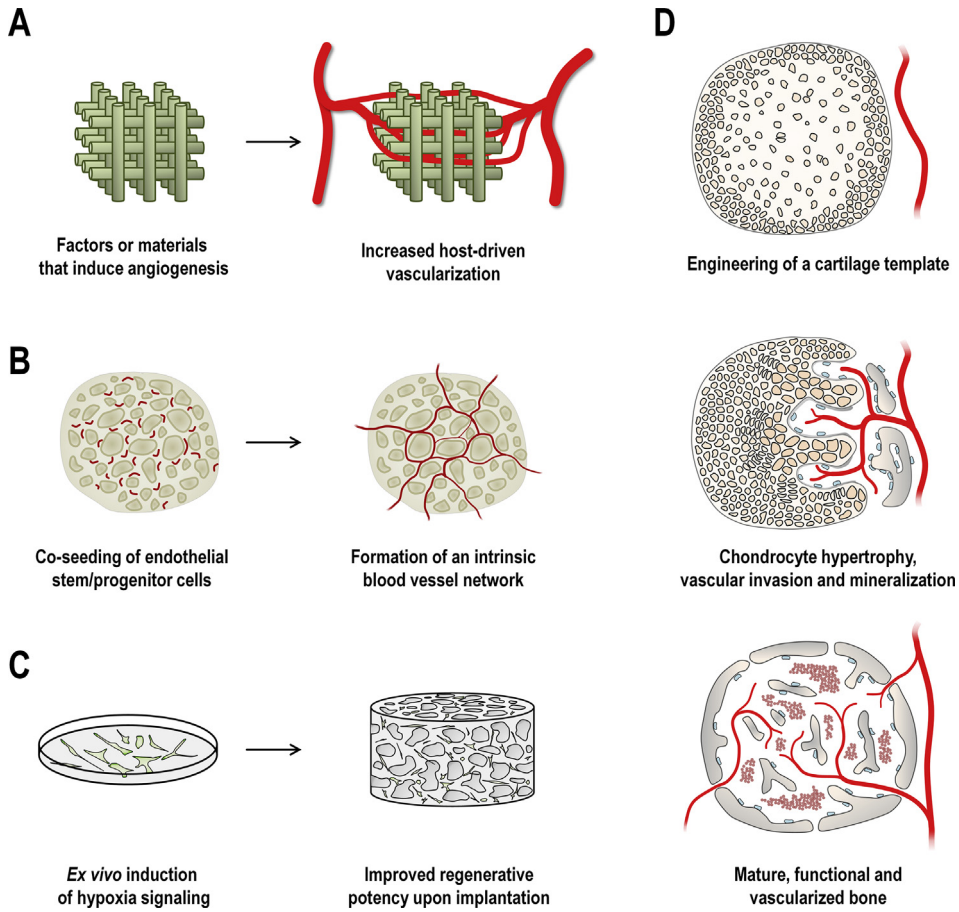

Fig. 2. Schematic overview of experimental strategies related to vascularization and hypoxia signalling that can enhance bone regeneration in a tissue-engineered construct. A. Application of angiogenic growth factors or adaptations in scaffold designs can increase vascularization by the host. B. Implantation of endothelial stem/progenitor cells may allow a vascular network to form from within the construct. C. Induction of hypoxia signalling prior to implantation can elicit a beneficial stress response that may improve therapeutic potency through enhanced survival, proliferation or differentiation of the implanted cells. D. Engineering of a cartilage template, as chondrocytes may survive better in an avascular environment. This approach may lead to bone formation through endochondral ossification.

2003). Interestingly, mesenchymal and endothelial cells show a positive interaction already during 2D culture via a mechanism that is not exclusively regulated by VEGF (Villars et al., 2000), indicating that mesenchymal cells may contribute a perivascular component that stabilizes the formed blood vessels (Melero-Martin et al., 2008; van Gastel et al., 2012).

In conclusion, increased vascularization can be obtained through the use of angiogenic growth factors, endothelial cells, and scaffold design. While each of these strategies individually has proven to be beneficial for the supply of oxygen to a bone tissue-engineered construct, a combination approach may be required to achieve a viable implant of clinically relevant size.

8. Modulating hypoxia signalling pathways as a cellular protection strategy

While many strategies are aimed to prevent hypoxia in a large tissue-engineered construct, hypoxia signalling itself can also be invoked more prominently to improve the therapeutic outcome of a tissue-engineered construct. This effect can be obtained through hypoxic preconditioning (Fig. 2C), inhibition of negative regulators of HIF-1α (PHDs and VHL) in cells prior to implantation, or direct overexpression of HIF. Hypoxia preconditioning is a cellular adaptive mechanism elicited by sublethal exposure of cells to low oxygen concentrations (Murry et al., 1986). The stress response that follows will protect cells against subsequent hypoxic insults, leading to improved function and survival through upregulation of anti-apoptotic and pro-angiogenic factors in, for example, stem cell-based treatment of myocardial infarction (Azarnoush et al., 2005; Hu et al., 2008), ischaemic disease in limb and brain (Bhang et al., 2011; Rey et al., 2011; Theus et al., 2008) or spinal cord injury (Oh et al., 2010).

For applications in bone tissue engineering, overexpression and small molecule-induced stabilization of HIF-1α have been demonstrated to increase angiogenesis and subsequent bone regeneration in tissue-engineered constructs (Ding et al., 2014; Zou et al., 2011), but it remains unexplored whether enhanced angiogenesis is the only contributing factor in these models. A recent study demonstrates that hypoxic preconditioning of bone marrow stromal cells enhances *in vivo* bone formation in a bone tissue-engineered construct, which was associated with increased collagen matrix abundance (Lee et al., 2015). Finally, *in vitro* data show that the incorporation of PHD or VHL inhibitors into the scaffold structure may lead to the stabilization of HIF in the seeded cells, which in turn can increase the expression of VEGF and promote the differentiation towards the osteogenic lineage. Examples include bioactive glass functionalized with dimethyloxallyl glycine (DMOG; (Wu et al., 2013)) which blocks PHDs, or with cobalt (Quinlan et al., 2015) which inhibits PHD activity. Because the small molecules used thus far to stabilize HIF-1α do not only target PHD specifically,

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one has to be cautious about off-target and unspecific effects.

Taken together, most studies on hypoxia signalling in bone tissue engineering mainly focus on angiogenesis, which is important for bringing oxygen and nutrients into the implant in order to sustain the viability of the implanted cells, and therefore the overall success of the implant. However, an increasing number of reports on other cell types show that hypoxia signalling can also affect the implanted cells directly, either through increasing cell survival, or through regulation of cellular proliferation and differentiation. Careful dissection of these processes will allow optimization of bone tissue engineering construct combinations with improved viability and regenerative potential.

9. Endochondral bone formation as a physiological adaptation against hypoxia

The studies discussed above focus largely on the generation of tissue-engineered constructs that promote bone formation through the intramembranous pathway. However, most of our bones form through the endochondral bone formation process during development. Recapitulating this process as a strategy for bone tissue engineering, known as developmental engineering (Lenas et al., 2009), may circumvent many of the problems that are related to the harmful hypoxia in tissue engineering constructs. In this approach, a cartilaginous template is engineered instead of implanting cell-based constructs that form bone directly (Fig. 2D). Indeed, implantation of a chondrogenic construct can spontaneously induce the subsequent formation of mineralized tissue, reminiscent of what is seen during endochondral bone formation (Harada et al., 2014; Tam et al., 2014; van Gastel et al., 2014; Weiss et al., 2012). Moreover, endochondral ossification can also be induced using cell-free implants obtained by devitalizing chondrogenic constructs, especially when matrix characteristics and the presence of growth factors to attract host cells are preserved (Bourgine et al., 2014). Furthermore, while the effect of hypoxia on osteogenic differentiation is not yet fully clear, the activation of hypoxia signalling in chondrocytes (Pfander et al., 2004) leads to higher expression of cartilage markers during *in vitro* culture (Shi et al., 2015; Strobel et al., 2010; Zhou et al., 2015), as well as improved collagen cross-linking during matrix synthesis (Makris et al., 2014) despite lowering of the proliferation of chondrocytes. In this way, engineering of endochondral bone tissue makes use of the physiological role of hypoxia signalling during bone development and the potential of chondrocytes to survive and differentiate in an avascular environment.

10. Conclusion & future perspectives

Bone tissue engineering has as its goal to mimic the repair process in bone, which in turn greatly resembles bone development. In bone development and repair, hypoxia signalling has a crucial role, and many studies have focussed on the link between angiogenesis and bone formation which is largely mediated by VEGF, a target gene of the HIF pathway. Increasing angiogenesis in a bone construct of clinically relevant size will favour bone formation, as survival, proliferation and differentiation of the implanted cells are dependent on sufficient oxygen and nutrient supply (Feng et al., 2013; van Gastel et al., 2012; Zou et al., 2011). However, because blood vessel formation is an inherently slow process, completely preventing harmful hypoxia through neoangiogenesis alone may prove difficult.

In addition to vascular effects, hypoxia signalling mediates responses that alter cell metabolism, survival and differentiation directly. Current literature points towards positive effects of hypoxia signalling on undifferentiated MSC during *in vitro* culture (Tsai et al., 2011; Volkmer et al., 2010), where it promotes the preservation of the proliferative and multipotency capacities of MSC, but the effect of hypoxia signalling on differentiating and mature osteoblasts has not been as conclusively determined. In other tissue models of regenerative MSC-based therapies, hypoxia signalling enhances the *in vivo* outcome (Huang et al., 2014; Rey et al., 2011) by improving cell survival through adapting energy, pH and redox balance. Whether these adaptations would also prove beneficial in a bone tissue engineering strategy remains to be shown. It is important to note that these cell-autonomous effects do not depend on slow, partially host-driven processes such as angiogenesis, but instead may protect the implanted cells against the stressful environment much more rapidly and continuously. Furthermore, the cell-protective and pro-angiogenic effects of hypoxia signalling are not mutually exclusive and may even be used in synergy to enhance tissue regeneration. Finally, while enhancing angiogenesis may require (co-)implantation of additional factors or cells to attract blood vessels, hypoxic preconditioning to enhance bone formation can be done through pre-treatment alone, without requiring further manipulations or additions to the tissue-engineered construct.

In conclusion, hypoxia signalling mediates a protective response consisting of a pro-angiogenic and a pro-survival component, where the benefit of the angiogenic response has been well described in bone tissue engineering. The effects on metabolism, survival and differentiation of the implanted cells themselves have, however, been much less studied, but still offer many exciting opportunities to improve the therapeutic potential of bone tissue engineering.

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